ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Stability Indicating Analytical Method Development and Validation for Related Substances for Letrozole Tablets by RP-HPLC

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ABSTRACT

A stability indicating HPLC method was developed and subsequently validated for the quantitation of letrozole and its related substances in tablets. As per the USP method the test concentration is 10ppm, too low to detect the impurities as per ICH guidelines. In addition, this matter was referred to USP for their comments. They recommended changing the test concentration to 100ppm. However, the test concentration was optimized at 200ppm. The optimized conditions for letrozole and its related substances were using gradient mode, Zodiac SIL 120-C18H (125*4.6mm, 5.0µm) column with Mobile phase A containing Milli-Q water and Mobile phase B containing Milli-Q water and Acetonitrile in the ratio of 30:70 at different time intervals at a flow rate 1.0mL/min. UV detection was performed at 230nm.The method is simple, accurate and economical method for analysis of related substances in Letrozole. The described method is linear over a range of about 0.0115µg/mL to 1.278µ g/mL for Letrozole and Letrozole related compound A and linearity range between 0.0114µg/mL to 0.632µ g/mL for impurity-D. The method precision for the determination of related impurities was below 2% RSD. The Percentage recoveries of known related impurities from dosage forms ranged from 97.14% to 101.4%. LOD and LOQ of all related impurities of Letrozole were established as 0.004µg/ml for LOD and 0.012µ g/ml for LOQ . The molecule is forced to all stress conditions such as acid, base, oxidation, heat and photolysis as per the recommendations of ICH guidelines. All degradants are well separated from the main analyte. The method is useful in the quality control of bulk manufacturing and also in pharmaceutical formulations.

Key words: Letrozole, USP, Validation, Stability indicating.

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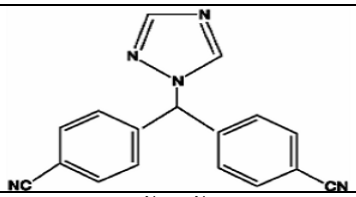
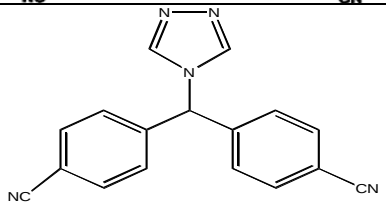
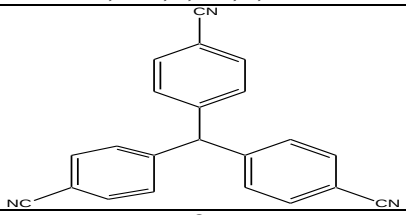
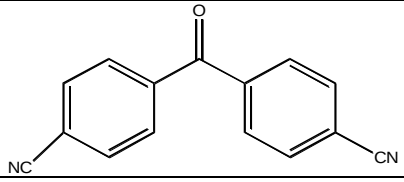
ISSN: 0975-8585

INTRODUCTION

Letrozole is Antineoplastic agent (Aromatase inhibitor), nonsteroidal competitive inhibitor of the aromatase enzyme system; it inhibits the conversion of androgens to estrogens**.** Chemical name is 4-[(4-cyanophenyl) (1H-1, 2,4-triazol-1-yl)methyl]benzonitrile. It is a white to yellowish crystalline powder, practically odorless, freely soluble in dichloromethane, slightly soluble in ethanol, and practically in soluble in water. Molecular formula is $C_{17}H_{11}N_5$ and Molecular weight is 285.31.The structural formula of Letrozole and related impurities were mention in Table 1.Existing literature reveals that Letrozole can be analysed by HPLC using electrochemical, fluorescence, mass spectrometry and UV for detection in bulk material and pharmaceutical forms. USP method employs amperometric electrochemical detection. Therefore, in proposed project a successful attempt has been made to develop simple, accurate and economic methods for analysis of related substances of Letrozole Tablets and validated. [1-4]

Table 1: Letrozole and its related impurities

NAME OF COMPOUND	STRUCTURE
LETROZOLE	
Letrozole Related Compound A (USP 29)	 $C_{17}H_{11}N_5$ Exact Mass: 285.10 Mol. Wt.: 285.30 C, 71.57; H, 3.89; N, 24.55
4, 4', 4''-methylidenetrisbenzonitrile (USP 29)	
Impurity A	

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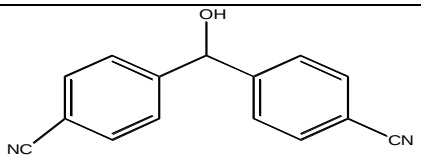
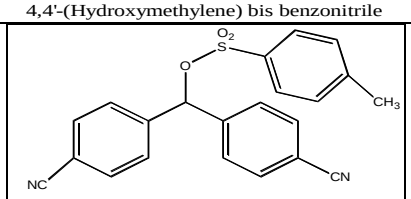
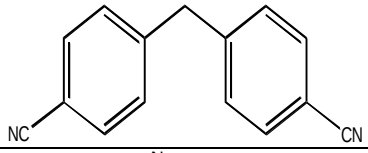
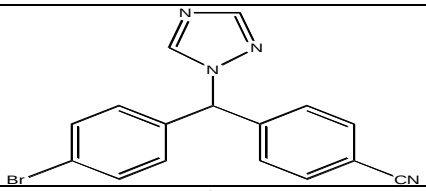
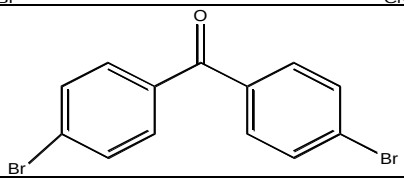
Impurity B	 4,4'-(Hydroxymethylene) bis benzonitrile
Impurity C	 Methanesulfonic acid bis-(4-cyano-phenyl)-methyl ester
Impurity D	
Impurity-E	
Impurity F	

METERIALS AND METHODS

Instrumentation

Agilent 1200 series equipped with UV detector, Semi Micro Balance(Sartorius ME235P),PH Meter(Thermo Electron Corporation Orion 2 Star), Sonicator (Ultrasonic Cleaner Power sonic 420), Centrifuge (Eppendorf Centrifuge 5810), Analytical Balance (Sartorius GE 212), Refrigerator (Samsung RT41MASW), Ultra micro balance (Sartorius ME235P), Vacuum oven (Wadegati; WIL-190).

Reagents

Letrozole working standard and related impurities were procured from Sun Pharmaceutical Industries Ltd. Acetonitrile (Merck HPLC grade), Water (Milli-Q water purification system), Sodium hydroxide (0.1N) (Merck GR), Hydrochloric acid (0.1N) (Merck GR), Hydrogen peroxide (3%) (Merck GR) ,0.45 µm Nylon filter (Axiva) ,0.45 µm PVDF filter (Axiva) [5, 6].

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Chromatographic conditions [7-15]

The following method was developed in Reverse Phase Hplc mode using, Zodiac SIL 120-5-C18 (125*4.6mm, 5.0µm) column with Mobile phase A containing Filtered and degassed Milli-Q water and mobile phase B-Filtered and degassed Milli-Q water and acetonitrile in the ratio of 30:70. The gradient programme includes T (time)/B (mobile phase B): 0/10, 3/10, 52/90, 60/90, 62/10, 70/10. The flow rate was 1ml/min and detection wavelength at 230nm. The whole operation was carried at 40°C temperature. Standard chromatogram of USP method and chromatogram obtained by following method was shown in figure 1, 2.

Figure 1: Typical Chromatogram of USP method

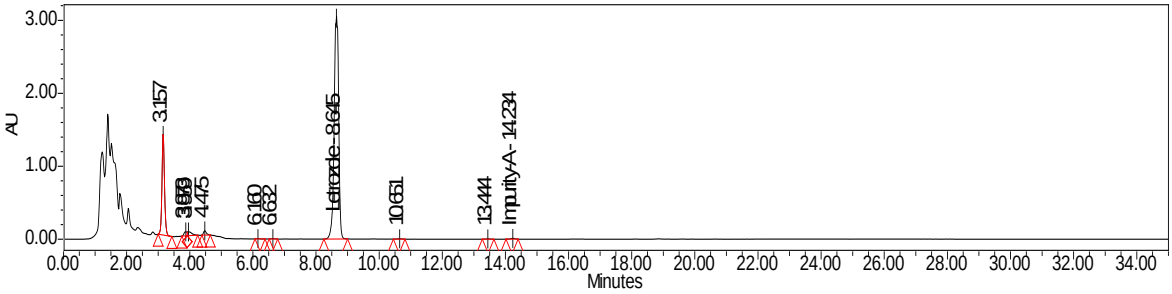
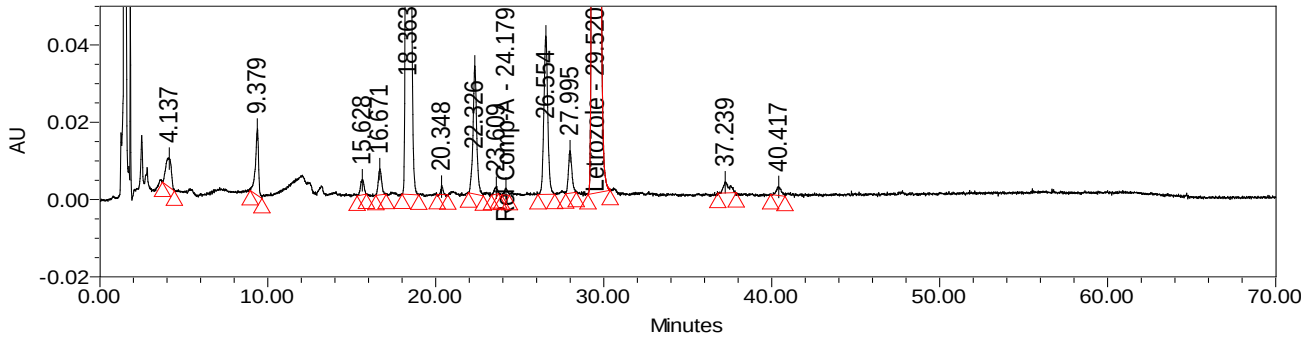


Figure 2: Typical Chromatogram of modified method



Standard & sample preparation:

Weigh accurately about 50mg of Letrozole working standard in 250ml volumetric flask, dissolve and dilute to volume with diluent and mix (200ppm).

Take 20 Tablets into a 250mL volumetric flask. Add 20mL of purified water and shake for 5 minutes to disintegrate the tablets. Add 75mL of acetonitrile shake for 30 minutes and sonicate for 5 min., then made volume with purified water. Centrifuge a portion of the solution at 3500 for 10minutes.

Placebo Preparation:

Take 20 placebo Tablets into a 250mL volumetric flask. Add 20mL of purified water and shake for 5 minutes to disintegrate the tablets. Add 75mL of acetonitrile shake for 30 minutes and sonicate for 5 min., then made volume with purified water. Centrifuge a portion of the solution at 3500 for 10minutes.

Forced degradation of Letrozole

Forced degradation of the sample was done to determine the intrinsic stability of the drug and to assess the stability of the developed method as per ICH guidelines.

Procedure

Weigh 10 tablets of Letrozole Tablets into a 20mL volumetric flask, add about 10mL diluent, sonicate for about 15 minutes in cool water then add 1mL of stress (0.1N HCl, 0.1N NaOH and water) and heat in a water bath for 30 minutes at 60°C. After specified time neutralize the solution. Cool the solution at room temperature and then dilute up to the mark with diluent. Centrifuge at 3000 rpm for 10 minutes, Filter through 0.2µ nylon membrane filter. An equivalent amount of placebo was treated in the similar conditions mentioned above and a Noised as per the proposed method. Peroxide degradation was carried out by using 5% H_2O_2. Thermal degradation of the sample was attempted by keeping the normal sample protected from light and the forced degradation sample in an oven at 80°C and analyzed at 24 and 48 hours. Photolytic degradation was done by exposing the sample to visible and UV light providing an overall illumination of1.2 million Lux hours and integrated ultraviolet energy of not less than 200 watt hours\sq meter. Blank of all the above conditions were first injected under the chromatographic condition mentioned under assay, followed by 6 replicates of the sample solution of forced degradation study to rule out the possible degradation of refluxing. Results are shown in Table 2, Figures3-10.

Table 2: Forced degradation results

Stress Condition	Drug Product			
	% Degradation	Letrozole		
		Purity angle	Purity threshold	Purity flag
Acid degradation	No Degradation	5.597	5.650	NO
Base degradation	2.02	4.570	5.997	NO
Peroxide degradation	45.23	2.961	4.508	NO
Water degradation	No Degradation	5.357	5.749	NO
UV degradation	No Degradation	6.009	8.882	NO
Thermal degradation	No Degradation	5.148	6.073	NO
Sunlight degradation	No Degradation	4.964	9.848	NO
Humidity degradation	No Degradation	5.366	5.597	NO

ISSN: 0975-8585

Figure 3: Un degraded Chromatogram

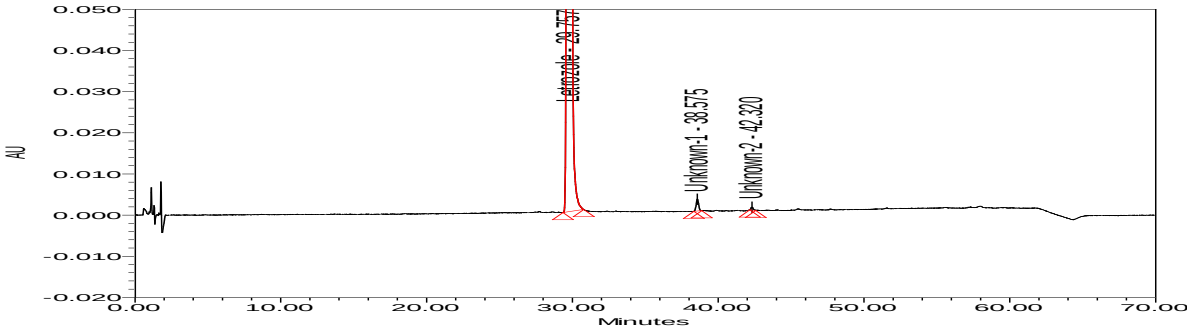


Figure 4: Acid Stress Chromatogram

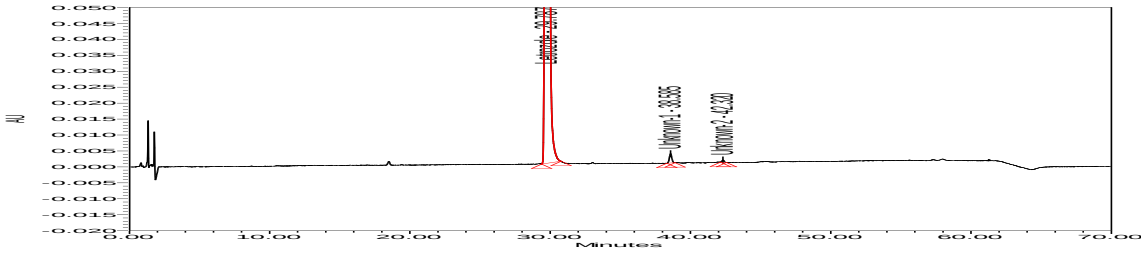


Figure 5: Base stress chromatogram

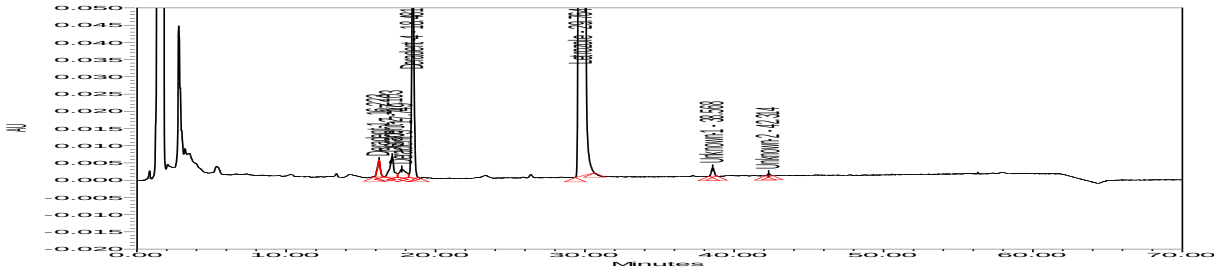


Figure 6: Peroxide stress Chromatogram

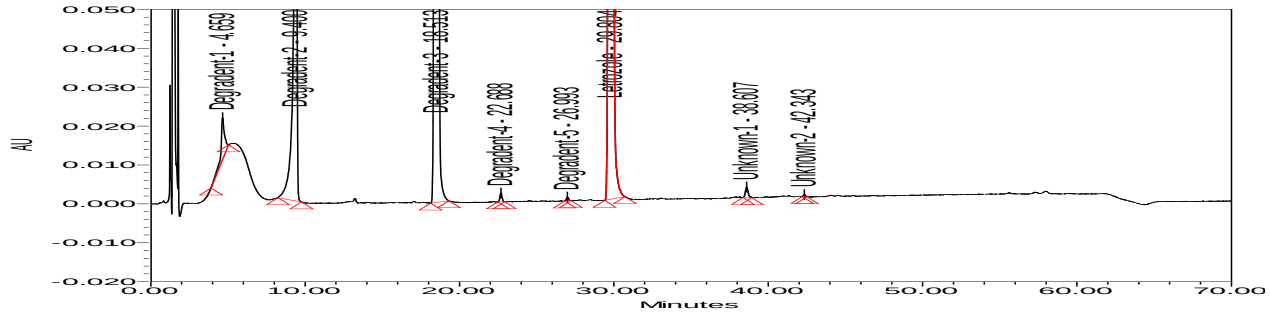


Figure 7: UV Light Stress Chromatogram

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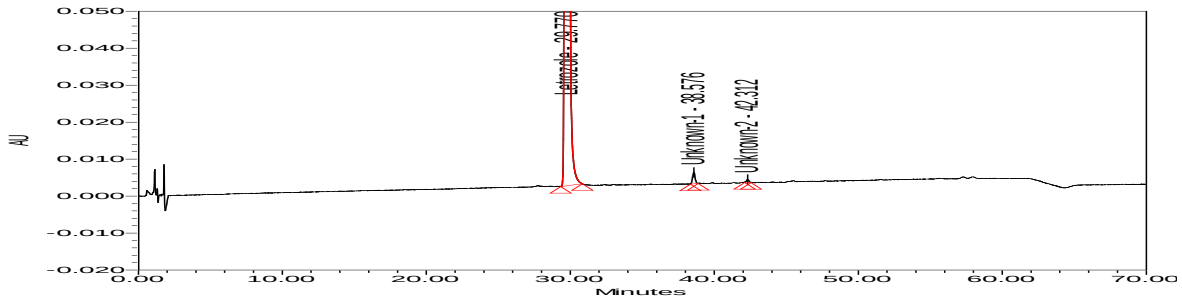


Figure 8: Sun Light Stress Chromatogram

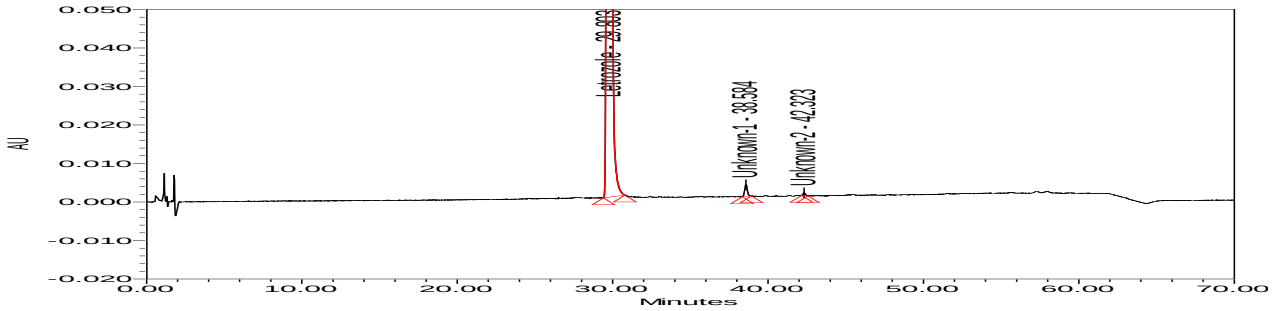


Figure 9: Thermal Stress Chromatogram:

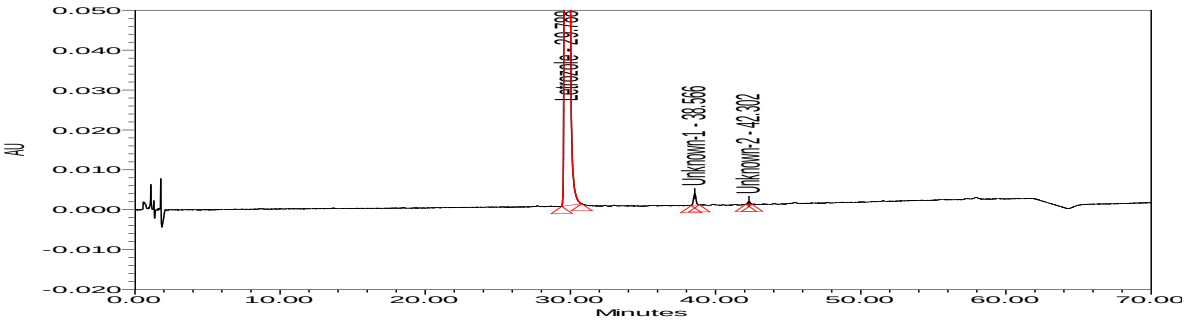
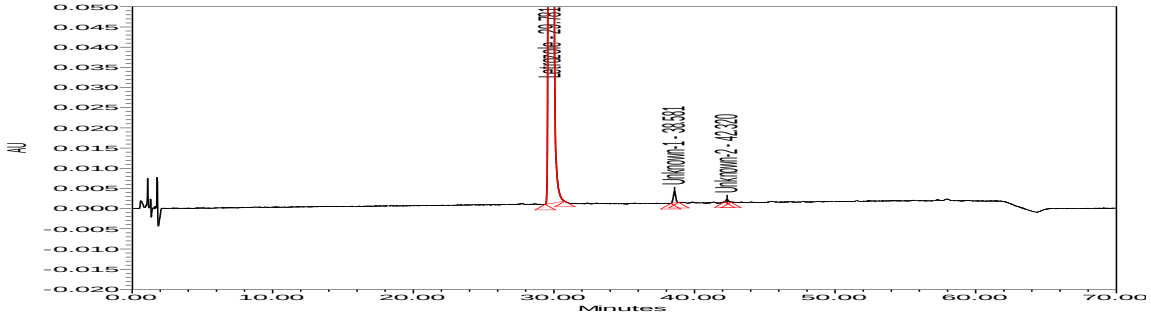


Figure 10: Water Stress Chromatogram



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METHOD VALIDATION [16-24]:

System suitability and system precision:

A Diluted Standard solution was prepared by using Letrozole working standard as per test method and was injected six times into the HPLC system. The system suitability parameters were evaluated from standard chromatograms and found to be within the limits. Calculate the %RSD from six replicate injections for Letrozole peak area. The %RSD for peak areas and system suitability was found to be within the limits. Results are shown in tables 3, 4 and figure 11.

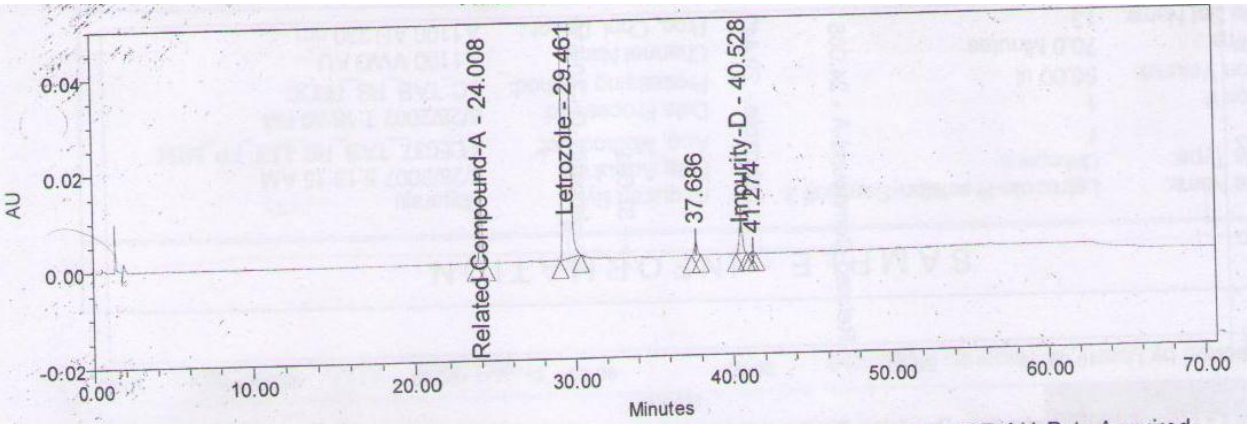
Table 3: System precision

Injection Number	Letrozole	Acceptance criteria
01	61016	The % Relative Standard Deviation of peak areas of Letrozole should not be more than 10.0
02	61350	
03	61530	
04	61636	
05	62116	
06	62267	
Average	61653	
%RSD	0.76	

Table 4: System suitability

System suitability	Observed value	Acceptance criteria
Similarity factor for diluted standard	0.99	0.98 to 1.02
USP theoretical plates for Letrozolepeak	141023	NLT 10000
Resolution between Letrozoleand Letrozolerelated compound-A	17.8	NLT 1.5
RRT of Letrozolerelated compound A	0.81	About 0.81

Figure 11: Chromatogram of Letrozole and its related impurities.



Specificity:

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The related substances of Letrozole Tablets were chromatographed individually and as a mixture added to sample and to placebo, to examine the interference with each other. Representative chromatograms of system suitability, standard, diluent, placebo, sample, and sample spiked with impurities, placebo spiked with impurities and individual impurity solutions as shown in fig 11.The results Peak is found to be homogeneous and there are no co-eluting peaks and relative retention time matches with method, hence indicating specificity of the method.

Limit of detection and Limit of quantitation

Prepared and injected the appropriate concentration of the Letrozole and its known impurities which will give signal to noise ratio of about 3 for LOD and about 10 for LOQ. Results are shown in table 5.

Table 5: LOD and LOQ values for Letrazole and it's impurities

Name of the Impurity	Test Name		Signal to Noise ratio		% at LOQ level
	Limit of Detection in ppm	Limit of Quantification in ppm	LOD	LOQ	
Letrozolerelated compound-A	0.004	0.012	3.4	10.3	0.006
Impurity-D	0.004	0.012	2.6	9.5	0.006
Letrozole	0.004	0.12	3.3	10.4	0.006

Table 6: LOQ precision for related compound –A and impurity-D

Sample No.	Related compound-A		Impurity-D		Letrozole	
	RRT	% Imp	RRT	% Imp	RT	% Imp
01	0.81	0.0060	1.38	0.0052	29.59	0.0066
02	0.81	0.0061	1.38	0.0051	29.61	0.0065
03	0.81	0.0061	1.38	0.0052	29.63	0.0066
04	0.81	0.0059	1.38	0.0053	29.62	0.0064
05	0.81	0.0061	1.38	0.0053	29.66	0.0065
06	0.81	0.0062	1.38	0.0052	29.67	0.0064
Average	NA	0.0060	NA	0.0052	NA	0.0065
%RSD	NA	1.5	NA	1.1	NA	1.6

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Table 7: LOQ accuracy for related compound –A and impurity-D

Sample No.	Letrozole Related compound-A			Impurity-D		
	'µg/mL' added	'µg/mL' Found	% Accuracy	µg/mL' added	'µg/mL' Found	% Accuracy
01	0.0115	0.0119	103.58	0.0114	0.0104	91.73
02	0.0115	0.0122	105.90	0.0114	0.0103	90.38
03	0.0115	0.0121	105.27	0.0114	0.0104	91.81
Average	NA	0.0121	104.9	NA	0.0104	91.3
%RSD	NA	--	1.14%	NA `	--	0.88%

Table 8: LOQ accuracy for Letrazole

Sample No.	Letrozole		
	'µg/mL' added	'µg/mL' Found	% Accuracy
01	0.0116	0.0124	107.63
02	0.0116	0.0122	105.61
03	0.0116	0.0123	106.84
Average	NA	0.0123	106.7
%RSD	NA	--	0.95%

For precision at LOQ as follows Prepared Letrozole and its known impurities solution at about LOQ concentration and injected six times into the HPLC as per the test method. Prepare the LOQ precision samples and inject into system and Calculated the % RSD for each known impurity and Letrozole. Results were shown in table 6 and accuracy at LOQ level results were shown in Tables 7, 8.

Accuracy

A known amount of Letrozole Tablets was taken into volumetric flask and spiked with known quantities of each named impurity at LOQ, 50%, 75%, 100%, 150% and 200% in triplicates. %Accuracy should be in the range of

a) 80-120% for LOQ level and impurity less than 0.05%
b) 85-115% for impurities between 0.05 – 0.50%
c) 90-110% for impurities between 0.51 – 2.0%
d) 95-105% for impurities more than 2.0%.Results shown in tables 9, 10 and figure 12.

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Linearity:

The linearity of response for each known impurity was determined in the concentration range of limit of quantitation to about 200% of specification limit for each known impurity. Acceptance criteria squared correlation coefficient not less than 0.99. The Correlation coefficient as shown in Tables 11, 12 and Figures 13, 14.

Table-9: Accuracy for Letrozole related compound A

Sample No.	Spike level	'µg/mL' added	'µg/mL' found (recovered)	Mean % recovery
1	50%	0.320	0.317	99.3
2	50%	0.320	0.316	
3	50%	0.320	0.318	
1	75%	0.479	0.499	102.4
2	75%	0.479	0.487	
3	75%	0.479	0.487	
1	100%	0.639	0.642	99.9
2	100%	0.639	0.637	
3	100%	0.639	0.636	
1	150%	0.959	0.963	101.3
2	150%	0.959	0.989	
3	150%	0.959	0.962	
1	200%	1.278	1.272	99.1
2	200%	1.278	1.262	
3	200%	1.278	1.265	

Table-10: Accuracy for Impurity-D

Sample no.	Spike level	'µg/mL' added	'µg/mL' found (recovered)	Mean % recovery
1	50%	0.158	0.150	95.3
2	50%	0.158	0.150	
3	50%	0.158	0.151	
1	75%	0.237	0.235	97.5
2	75%	0.237	0.230	
3	75%	0.237	0.288	
1	100%	0.316	0.306	96.2
2	100%	0.316	0.304	
3	100%	0.316	0.303	
1	150%	0.474	0.466	99.3
2	150%	0.474	0.479	
3	150%	0.474	0.467	
1	200%	0.632	0.617	97.4
2	200%	0.632	0.614	
3	200%	0.632	0.615	

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Figure 12: Chromatogram of Accuracy at 50%level

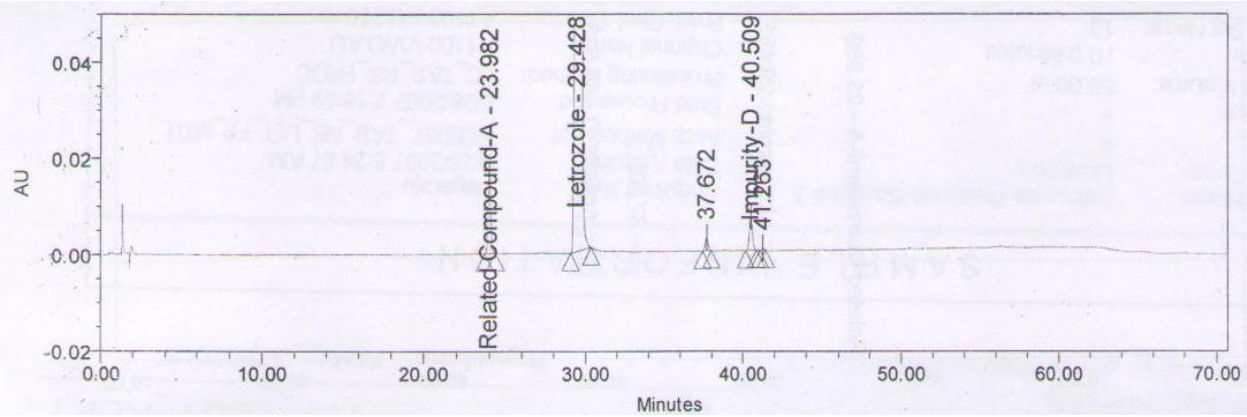

Table 11: Linearity for Letrozole Related compound-A

Spike Level	Average 'µg/ml ' Added	Average 'µg/ml ' Found
LOQ	0.0115	0.0121
50%	0.320	0.317
75%	0.479	0.491
100%	0.639	0.638
150%	0.959	0.971
200%	1.278	1.266
Coefficient of correlation (r)		0.999

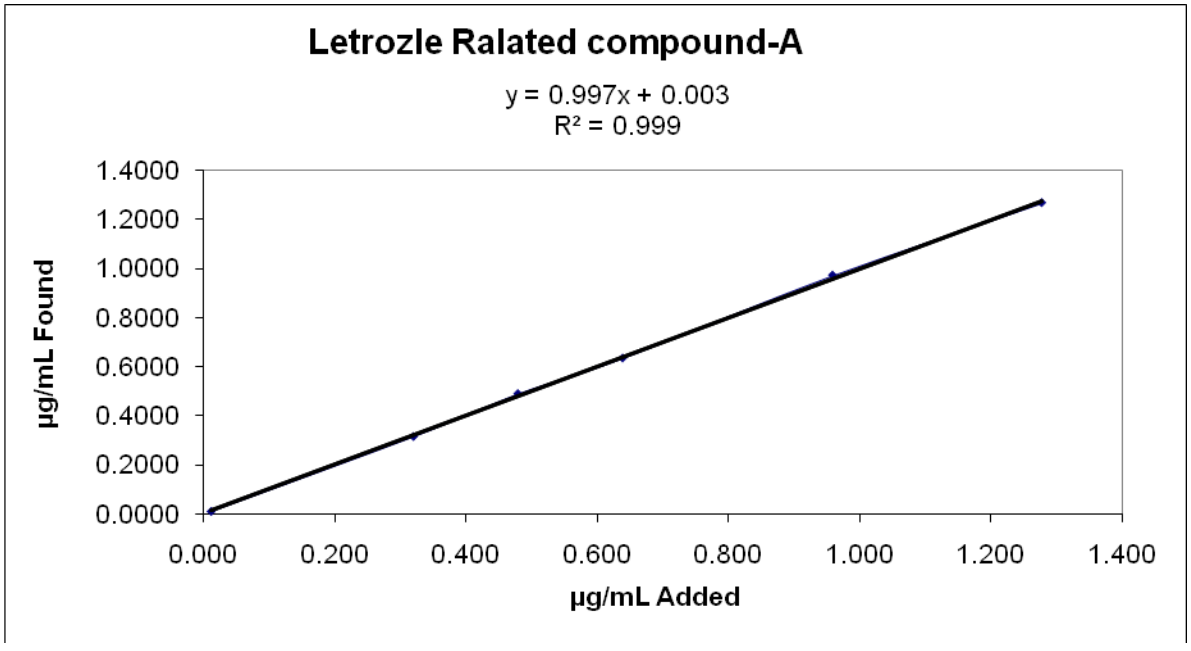

Figure 13: Linearity curve for Letrozole Related compound-A

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Table 12: Linearity for Letrozole Impurity-D

Spike Level	Average 'µg/ml ' Added	Average 'µg/ml ' Found
LOQ	0.0114	0.0104
50%	0.158	0.151
75%	0.237	0.231
100%	0.316	0.304
150%	0.474	0.470
200%	0.632	0.616
Coefficient of correlation (r)		0.999

Figure 14: Linearity curve for Letrozole impurity-D

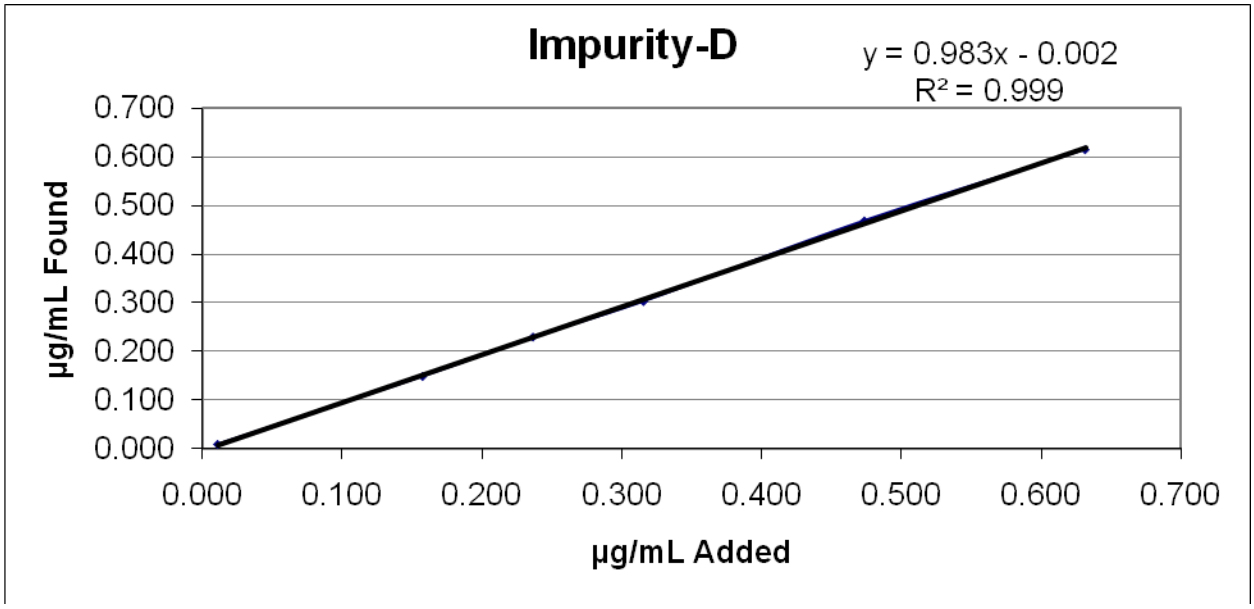

Table 13: Intermediate precision (ruggedness)

System suitability	Observed value		Acceptance criteria
	System-1	System-2	
Similarity factor for diluted standard	0.99	0.99	**0.98 to 1.02**
USP theoretical plates for Letrozole peak	141023	154676	**NLT 10000**
Resolution between Letrozole and Letrozole related compound-A	17.8	19.6	**NLT 1.5**
RRT of Letrozole related compound A	0.81	0.81	**About 0.81**

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Table 14: For first analyst data (ruggedness)

Sample No.	Letrozole Related compound-A		Impurity-D		Total Impurities
	RRT	% Impurity	RRT	% Impurity	
01	0.81	0.308	1.39	0.153	0.527
02	0.81	0.320	1.39	0.159	0.549
03	0.81	0.321	1.39	0.159	0.549
04	0.81	0.313	1.39	0.156	0.536
05	0.81	0.315	1.39	0.156	0.540
06	0.81	0.312	1.39	0.155	0.534
Average	0.81	0.315	1.39	0.156	0.539
% RSD	NA	1.6%	NA	1.6%	1.6%

Table 15: For second analyst data (ruggedness)

Sample No.	Letrozole Related compound-A		Impurity-D		Total Impurities
	RRT	% Impurity	RRT	% Impurity	
01	0.82	0.317	1.38	0.153	0.538
02	0.82	0.316	1.38	0.153	0.536
03	0.82	0.315	1.38	0.152	0.534
04	0.82	0.316	1.38	0.153	0.536
05	0.82	0.316	1.38	0.153	0.537
06	0.81	0.315	1.38	0.152	0.535
Average		0.32		0.15	0.536
% RSD		0.2		0.2	0.3

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Table 16: Effect of variation in mobile phase-B composition:

System suitability Parameters	Mobile phase composition			Acceptance criteria
	90% of Acetonitrile (630:370 v/v)	100%of Acetonitrile (700:300v/v)	110% of Acetonitrile (770:230v/v)	
Similarity factor for diluted standard	0.98	1.00	0.99	0.98 to 1.02
USP theoretical plates for Letrozole peak	139978	138075	137258	NLT 10000
Resolution between Letrozole and Letrozole related compound-A	17.5	17.9	17.0	NLT 1.5
RRT of Letrozole related compound A	0.82	0.81	0.82	About 0.81
RRT of Impurity-D	1.35	1.37	1.34	NA

Table 17: Effect of variation of flow rate

System suitability Parameters	Flow rate Variation			Acceptance criteria
	0.8mL/min	1.0mL/min	1.2mL/min	
	0.99	1.00	0.99	
	132781	138075	128807	
	16.9	17.8	17.6	
	0.82	0.81	0.81	
	1.35	1.4	1.43	

Table 18: Effect of Flow rate

Name of Impurity	with 0.2ml/min less		As such Flow Rate		0.2ml/min More	
	Resolution	RRT	Resolution	RRT	Resolution	RRT
USP-A	0	0.82	0	0.82	0	0.81
Letrozole	14.28	1.0	16.28	1.0	12.78	1.0
Impurity-B	2.63	1.04	2.97	1.04	2.19	1.04
Impurity-A	15.26	1.25	17.55	1.26	13.21	1.27
Impurity-F	18.46	1.95	21.63	1.97	18.57	1.99

ISSN: 0975-8585

Fig-15: 0.2ml/min Flow Rate Less

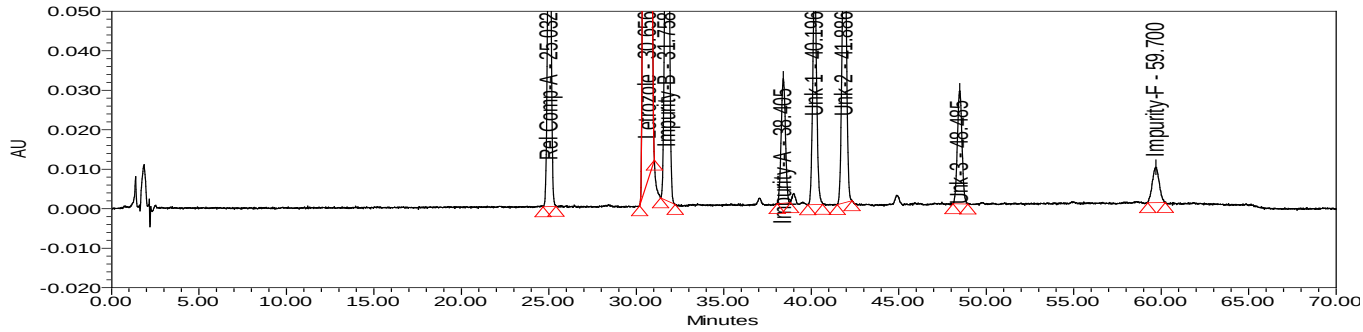

Fig-16: 0.2ml/min Flow Rate more

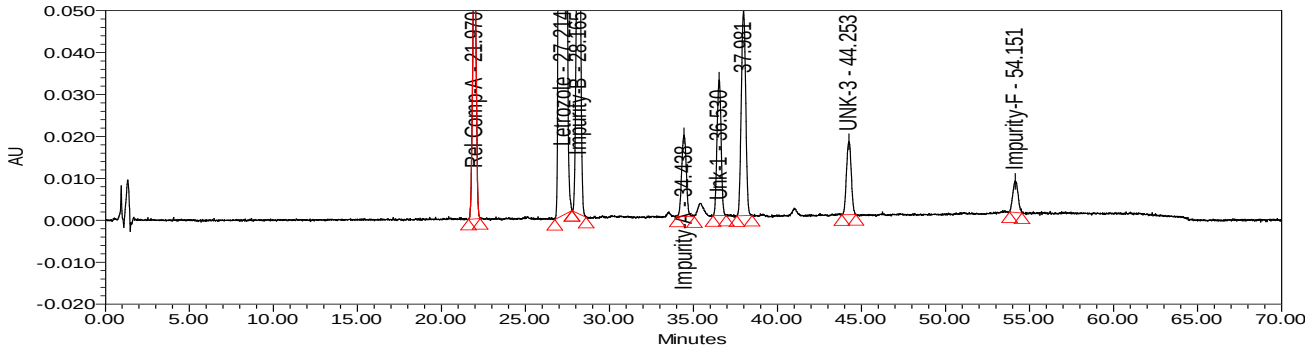

Table 19: Effect of column temperature variation

System suitability Parameters	Column Temperature Variation			Acceptance criteria
	35°C	40°C	45°C	
Similarity factor for diluted standard	0.99	1.00	0.99	**0.98 to 1.02**
USP theoretical plates for Letrozole peak	139655	138075	126032	**NLT 10000**
Resolution between Letrozole and Letrozole related compound-A	17.4	17.9	17.6	**NLT 1.5**
RRT of Letrozole related compound A	0.81	0.81	0.81	**About 0.81**
RRT of Impurity-D	1.38	1.40	1.38	**NA**

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Table 20: Effect Column Temperature

Name of Impurity	with 5°C less		As such Column Temp.		with 5°C more	
	Resolution	RRT	Resolution	RRT	Resolution	RRT
USP-A	0	0.82	0	0.82	0	0.81
Letrozole	15.9	1.0	16.28	1.0	14.77	1.0
Impurity-B	3.13	1.04	2.97	1.04	2.4	1.04
Impurity-A	17.54	1.26	17.55	1.26	15.12	1.26
Impurity-F	20.86	1.95	21.63	1.97	20.26	1.98

Fig-17: 5°C Column Temp. Less

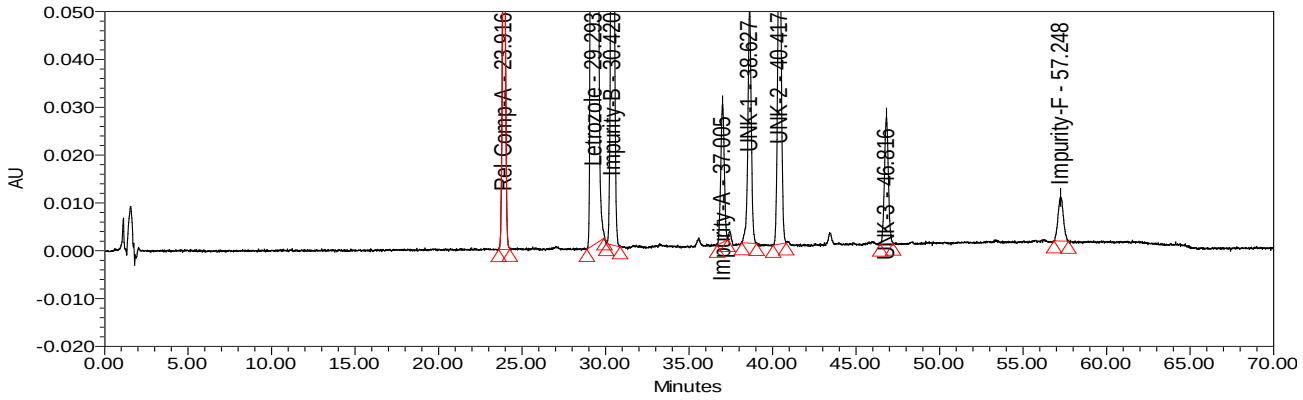

Fig-18: 5°C Column Temp. More

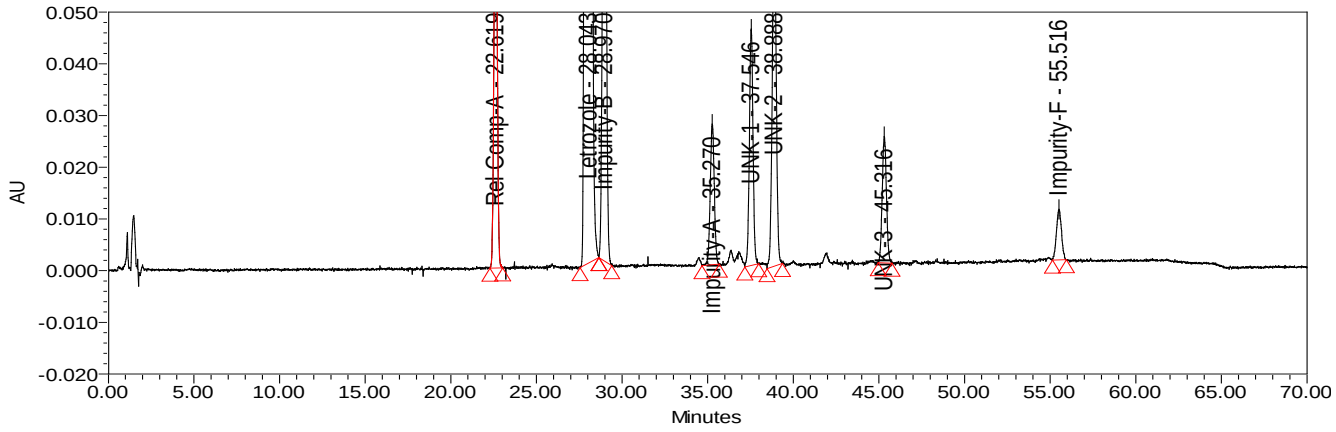

Table 21: Bench top Stability of standard Preparation

Time in days	Letrozole Standard	
	%	Diff. From initial
Initial	99.79	NA
01	99.8	0.01
02	99.8	0.01

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Table 22: Bench top Stability of standard Preparation

Sample No.	Letrozole related compound-A				Impurity-D				Total impurities			
	% Impurity		Diff. From initial		% Impurity		Diff. from initial		% of impurity		Diff. From initial	
	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Initial	0.317	0.316	NA	NA	0.153	0.153	NA	NA	0.538	0.536	NA	NA
01	0.319	0.315	0.002	0.001	0.154	0.152	0.001	0.001	0.539	0.532	0.001	0.004
02	0.322	0.315	0.005	0.001	0.156	0.153	0.003	0.000	0.546	0.536	0.008	0.000

Intermediate precision (ruggedness):

The intermediate precision of the method was determined by analyzing a sample prepared as per the method and different analysts on different days six samples of Letrozole Tablets were analysed as per the method. Each named impurity and total impurities were calculated on these samples. The test results were not affected. Results were shown in tables13, 14, and 15.

Robustness:

To determine the robustness of the developed method experimental conditions were purposely altered and Theoretical plates for Letrozole peak from first chromatogram of standard not less than 3000,Tailing factor for Letrozole peak from chromatogram of standard not more than 2.0 and % RSD for replicate standard injections not more than 5.0.Results were shown in Tables16-20 and Figures15-18

Solution Stability:

Solution stability was checked and Sample solution spiked with impurities is found to be stable up to 1440 minutes at 10°C.% difference of response from initial for each known impurity >0.1% not more than 15 and total impurities not more than10.Results were shown in Tables 21, 22.

CONCLUSION

A high performance liquid chromatography method was developed and validated for the determination of the related substances for Letrozole in Letrozole Tablets. The proposed method was found to be good results for specificity, linearity, precision, intermediate precision, and accuracy, stability in analytical solution, robustness and degradation studies. Therefore the method is suitable for its intended use for commercialization.

ISSN: 0975-8585

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